From the Ludwig Institute for Cancer Research Ldt and
The Department of Cell and Molecular Biology,
Karolinska Institutet, Stockholm, Sweden

# Vascular Metabolomics –

## Role of VEGF-B in fatty acid uptake and metabolic disease

**Carolina Hagberg**


Karolinska Institutet

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*To My Love and to Happy Times ahead* ♥

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Diabetes är en sjukdom där patienterna inte kan använda sitt blodsocker på ett normalt sätt på grund av problem med hormonet insulin. Diabetiker delas upp i ungdomsdiabetiker (typ 1) och vuxendiabetiker (typ 2). Till skillnad från ungdomsdiabetiker som helt saknar insulin, kan vuxendiabetiker producera eget insulin. Normalt ger insulin en signal till musklerna att de skall ta upp socker från blodet efter en måltid. Hos vuxendiabetiker saknar insulinet verkan i musklerna, vilket brukar kallas att musklerna har blivit insulinresistenta. Då insulinsignaleringen inte fungerar kan socker inte tas upp av musklerna, och de drabbade får högt blodsocker som följd. Ny forskning har visat att musklernas insulinresistens uppkommer till följd av för mycket fett inne i musklerna. Fettet hindrar att blodsockret används. Det vore därför önskvärt att minska fettet inne i musklerna och hellre lagra det i fettvävnaden.

Musklernas fettupptag har tidigare studerats grundligt. Däremot känner man inte till hur fetterna transporteras från blodcirkulationen igenom blodkärlen fram till musklerna. Man vet inte heller vad som *reglerar* blodkärlens fettupptag, eller vilka fettransportörer som är involverade. Denna avhandling visar att blodkärlen har en roll i fettupptaget, och att en minskning av blodkärlens fettupptag skyddar mot vuxendiabetes.

**Delarbete 1** beskriver blodkärlens roll i fettupptaget från blodcirkulationen till musklerna. Vi studerar ett protein som kallas VEGF-B, som produceras av musklerna och skickas av dessa till närliggande blodkärl. VEGF-Bs funktion har tidigare varit okänd. Vi visar att rollen för VEGF-B är att berätta för blodkärlen att musklerna behöver mer näring i form av fetter. På det sättet kan musklerna troligen få tillräcklig näring då de arbetar, och samtidigt skyddas mot för mycket fettupptag. Stimulering av blodkärl med VEGF-B ledde till att blodkärlen tillverkade fler fettransportörer, och därmed tog upp mer fetter. Då vi hämmade VEGF-B eller fettransportörerna upphörde fettupptaget. Vi studerade även en musstam som saknar VEGF-B (Vegfb$^{-/-}$ möss). Dessa möss hade lägre fettupptag till muskler och hjärta, och därmed även minskad ansamling av fett inne i musklerna. Istället tog Vegfb$^{-/-}$ mössen upp mer socker från blodcirkulationen. Sammanfattningsvis visar studien att VEGF-B kan kontrollera blodkärlens fettransport, och att inhibering av VEGF-B minskar mängden fett i musklerna.

**Delarbete 2** visar hur en begränsning av VEGF-B kan skydda mot utvecklingen av diabetes. För att studera diabetes hos möss använde vi oss av en musstam med möss som blir väldigt feta och parallellt utvecklar vuxendiabetes (db/db möss). Vi korsade db/db mössen med Vegfb$^{-/-}$ mössen. Vi testade även att behandla db/db möss med en antikropp som binder till och hämmar VEGF-B. Både musstamskorsningen och antikroppsbehandlingen ledde till lägre fettinnehåll i hjärtmuskulaturen. Som följd av detta minskade insulinresistensen och db/db mössen började använda mer blodsocker. Dessutom visar vi att korrigering av insulinresistens i hjärta och muskler inte bara leder till lägre blodsocker, utan även till lägre blodfetter och bättre kolesterolvärden. Studien visar att utan VEGF-B fungerar blodkärlen som ett effektivt hinder mot alltför högt fettupptag till musklerna. Behandling med antikroppar som begränsar VEGF-B har därmed potential att minska insulinresistens och vuxendiabetes.

# ABSTRACT

The incidence of type 2 diabetes and the metabolic syndrome is rapidly increasing among both adults and children worldwide. Type 2 diabetes is strongly associated with obesity and ectopic lipid accumulation. Recent research has shown that peripheral insulin sensitivity is directly impaired by excessive lipid deposition within tissues. Therefore, it would be highly beneficial to be able to control lipid uptake and accumulation in organs prone to developing insulin resistance. The role of the vasculature as a controlling barrier for FA uptake has not previously been explored in detail.

In **Paper I**, we describe an unexpected role for Vascular Endothelial Growth Factor B (VEGF-B) in the control and endothelial targeting of fatty acids to heart and muscle. VEGF-B signals in a paracrine fashion through its receptors present on vascular endothelial cells. We show that the expression of *Vegfb* is tightly co-regulated with the expression of nuclear-encoded mitochondrial genes. VEGF-B signalling to the endothelium upregulates the mRNA and protein levels of Fatty Acid Transport Proteins (FATPs). Increased vascular FATP-levels leads to subsequent uptake and transport of long chain fatty acids across the endothelium. Mice lacking VEGF-B, or its receptors, have lower endothelial expression of FATPs and show less accumulation of lipid droplets within peripheral tissues. We conclude that VEGF-B is part of a novel regulatory mechanism, whereby endothelial lipid uptake and mitochondrial lipid usage is tightly coordinated.

In **Paper II**, we show that inhibition of VEGF-B signalling protects against the development of insulin resistance in the *db*/*db* mouse model of type 2 diabetes. Genetic deletion of either one, or both, copies of *Vegfb* in *db*/*db* mice significantly reduces cardiac lipid deposition and leads to increased glucose usage. The *db*/*db*//*vegfb*$^{-/-}$ mice are protected against the development of hyperglycaemia, glucose intolerance and triglyceridemia. Pre-diabetic *db*/*db* mice receiving neutralising anti-VEGF-B antibodies similarly do not develop hyperglycaemia or triglyceridemia. Anti-VEGF-B treatment of mice with established diabetes prevents a further increase in blood glucose levels. The study shows that the endothelium can act as an efficient barrier against excessive nutrient uptake, even in a pathological context. Based on these results, we propose that targeting VEGF-B could be a future approach for treating peripheral insulin resistance and type 2 diabetes.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACS(V)L | (Very) Long-chain Acyl CoA Synthetase |
| ATP | Adenosine triphosphate |
| BAT | Brown Adipose Tissue |
| BODIPY-FA | 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-S-indacene-3-dodecanoic acid |
| CYCS | Cytochrome C, somatic |
| DAG | Diacylglycerol |
| Db/db | Diabetic mouse model lacking a functional leptin receptor |
| ECs | Endothelial Cells |
| ERRα | Estrogen-Related Receptor alpha |
| FAs | Fatty Acids |
| FABP | Fatty Acid Binding Protein |
| FATP | Fatty Acid Transport Protein |
| GPIHBPI | Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 |
| GTT | Glucose Tolerance Test |
| HFD | High Fat Diet |
| HIF1a | Hypoxia Inducible Factor 1 alpha |
| HSP | Heparan Sulfate Proteoglycans |
| IR | Insulin Resistance |
| IRS | Insulin Receptor Substrate |
| LCFA | Long-chain Fatty Acid (13-21 carbon aliphatic tails) |
| LPL | Lipoprotein Lipase |
| MAPK | Mitogen-activated Protein Kinase |
| NDUFA5 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5 |
| NEFA | Non-esterified Fatty Acid (free fatty acid) |
| NO, eNOS | Nitric oxide, Endothelial NO synthase |
| NRP | Neuropilin |
| ORO | Oil Red-O, stain for neutral lipids |
| OXPHOS | Genes coding for proteins within the oxidative phosphorylation |
| PET | Positron Emission Tomography |
| PGC1α | PPARgamma co-activator 1 alpha |
| PI3K | Phosphatidylinositol 3-kinase |
| PKC | Protein Kinase C |
| PlGF | Placental Growth Factor |
| PM | Plasma Membrane |
| PPARγ | Peroxisome-proliferator activated receptor gamma |
| SHR | Spontaneously Hypertensive Rat |
| SNARE | Soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor |
| T1D | Type 1 Diabetes, juvenile or insulin-dependent diabetes |
| T2D | Type 2 Diabetes, adult-onset or non-insulin dependent diabetes |
| TCA cycle | Tricarboxylic Acid Cycle/Citric acid cycle |
| TGs | Triglycerides |
| TK | Tyrosine Kinase domain |
| VEGF(R) | Vascular Endothelial Growth Factor (Receptor) |
| (V)LDL | (Very) Low density lipoprotein |
| WAT | White Adipose Tissue |

# LIST OF ORIGINAL PUBLICATIONS

**This thesis is based on the following papers that will be referred to in the text by their roman numbers:**

I. **Hagberg CE**, Falkevall A, Wang X, Larsson E, Huusko J, Nilsson I, van Meeteren LA, Samen E, Lu L, Vanwildemeersch M, Klar J, Genove G, Pietras K, Stone-Elander S, Claesson-Welsh L, Ylä-Herttuala S, Lindahl P, Eriksson U (2010). Vascular endothelial growth factor B controls endothelial fatty acid uptake. *Nature* 464(7290):917-21.

II. **Hagberg CE**, Mehlem A, Falkevall A, Muhl L, Ortsäter H, Samen E, Lu L, Scotney P, Nash A, Stone-Elander S, Sjöholm Å, Eriksson U (2011). Targeting VEGF-B is a novel treatment of insulin resistance and type-2 diabetes. *Manuscript.*

**Other publications not included in the thesis:**

Albrecht I, Kopfstein L, Strittmatter K, Schomber T, Falkevall A, **Hagberg CE**, Lorentz P, Jeltsch M, Alitalo K, Eriksson U, Christofori G, Pietras K (2010). Suppressive effects of vascular endothelial growth factor-B on tumor growth in a mouse model of pancreatic neuroendocrine tumorigenesis. *PLoS One* 5(11):e14109.

Lähteenvuo JE, Lähteenvuo MT, Kivelä A, **Rosenlew C**, Falkevall A, Klar J, Heikura T, Rissanen TT, Vähäkangas E, Korpisalo P, Enholm B, Carmeliet P, Alitalo K, Eriksson U, Ylä-Herttuala S (2009). Vascular endothelial growth factor-B induces myocardium-specific angiogenesis and arteriogenesis via vascular endothelial growth factor receptor-1- and neuropilin receptor-1-dependent mechanisms.
*Circulation* 119(6):845-56.

Diesen C, Saarinen A, Pihko H, **Rosenlew C**, Cormand B, Dobyns WB, Dieguez J, Valanne L, Joensuu T, Lehesjoki AE (2004). POMGnT1 mutation and phenotypic spectrum in muscle-eye-brain disease. *J Med Genet* 41(10):e115.

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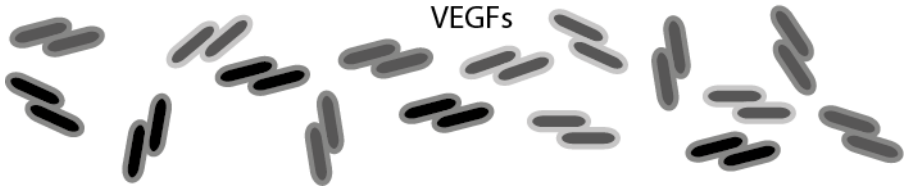


# 1 INTRODUCTION

Blood vessels are the highways of the body, transporting nutrients and oxygen to the most distant tissues and cells. Oxygen is used during mitochondrial oxidative phosphorylation to convert the energy from the nutrients into energy-rich ATP that the cells can utilise. Although tissue oxygenation has been extensively studied by vascular biologists[1,2] (Chapter I), little is known of the role of the vasculature in fatty acid (FA) transport and delivery[3,4] (Figure 1 and Chapter 2). However, it has become clear that excessive delivery of nutrients to peripheral cells can be deleterious to cellular function, and cause metabolic dysfunction and related diseases[5] (Chapter 3).

New blood vessels are formed from existing ones through a process called angiogenesis. Members of the Vascular Endothelial Growth Factor (VEGF) family are crucial players in both developmental and adult physiological and pathological angiogenesis[6]. Therefore, it was highly unexpected that a member of the family, VEGF-B, had a restricted angiogenic role[7,8], and was not regulated by tissue hypoxia[9].

The discovery that VEGF-B has a role in FA delivery to peripheral tissues is the central theme of this thesis (Paper I). The work describes a novel biological role for a member of the VEGFs, and shows that the vasculature participates in both the regulation and delivery of FAs to the tissue. Moreover, inhibition of VEGF-B-mediated FA uptake in diabetic *db*/*db* mice protects the mice against the development of metabolic disease (Paper II). Based on our data, we propose that VEGF-B is part of a novel regulatory cycle, coordinating mitochondrial lipid usage to lipid uptake. In retrospective, it is not surprising that nature uses members of the VEGF family for efficient delivery of both oxygen and of nutrients to the tissues.

*Vascular metabolomics* studies how vascular growth and endothelial cell (EC) physiology can impact the energy metabolism of the parenchymal cells (Chapter 4). Although this thesis focuses on the VEGFs and FA transporters, it will hopefully help to inspire additional new thinking around similar themes. Happy reading!

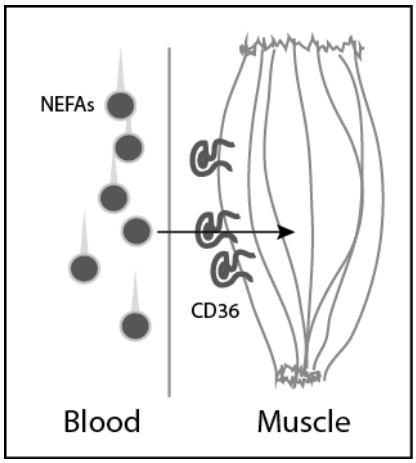



*Figure 1. Traditional view of FA transport.* The major rate-limiting step in FA uptake was thought to occur at the muscle sarcolemma, whereas the endothelium did not impose a barrier for lipid transport. NEFAs, non-esterified FAs

## 1.1 VEGFs IN HEALTH AND DISEASE

*"In my more grandiose moments, I think of a day to come when it may be possible to use angiogenesis inhibitors as an adjunct to conventional therapy"*

Judah Folkman, GHA Clowes Memorial Award Lecture 1986[10]

Vessels are formed as a primary vascular plexus through the process of vasculogenesis during early embryonic development[11]. The undifferentiated vessels undergo extensive angiogenic remodelling through growth, branching, sprouting and pruning, and form a mature organised network consisting of arteries, veins and capillaries. Although adult physiological angiogenesis is restricted, pathological angiogenesis is a key step in several disorders, most notably tumour development and macular degeneration in the retina[6]. Today pharmacological tools that inhibit pathological vessel growth have been developed[12]. Many of these tools target members of the VEGF family[6,13,14].

### 1.1.1 VEGF ligands and receptors

The VEGF family of growth factors are key mediators of vasculogenesis and angiogenesis both during embryonic development and in adult physiology and pathology[15-17]. The family includes five mammalian ligands, VEGF-A, -B, -C, -D and Placental Growth Factor (PlGF), which all occur in different splice variants[16]. They bind in a partially overlapping pattern to three tyrosine kinase (TK) receptors, VEGF receptor-1, -2 and -3 (VEGFR1-3)[15,17] (Figure 2). Co-receptors have been identified and include Neuropilin-1 and -2 (NRP1-2) and heparan sulphate proteoglycans (HSPs)[15,18]. The additional non-mammalian members of the VEGFs, found in parapoxvirus (VEGF-E) and snake venom (VEGF-F), will not be discussed within this thesis work[6].

The VEGF-ligands are secreted as ~40 kDa large dimeric glycoproteins, which signal in a paracrine fashion to VEGFRs[19,20]. They preferentially form homodimers, but VEGF-A/PlGF and VEGF-A/VEGF-B heterodimers have been isolated[21,22]. Binding of growth factors to their receptors is mediated by the VEGF-homology domain, consisting of eight conserved cysteine-residues[15,23]. Binding induces receptor dimerisation, and subsequent signalling inside the cell. All three VEGFRs are required for normal development, and knock-out mice lacking any of the receptors die prenatally[15,23].

#### VEGF-C and VEGF-D and VEGFR3

VEGF-C, VEGF-D and VEGFR3 are all implicated in the formation and maintenance of lymph-endothelial vasculature[23]. Mice lacking one or both copies of *Vegfc* develop oedema, and *Vegfc*$^{-/-}$ and *Vegfr3*$^{-/-}$ mice die during development[15,23]. The *Vegfd*$^{-/-}$ mice are healthy and fertile[15]. These VEGFs will not be further discussed in this thesis.

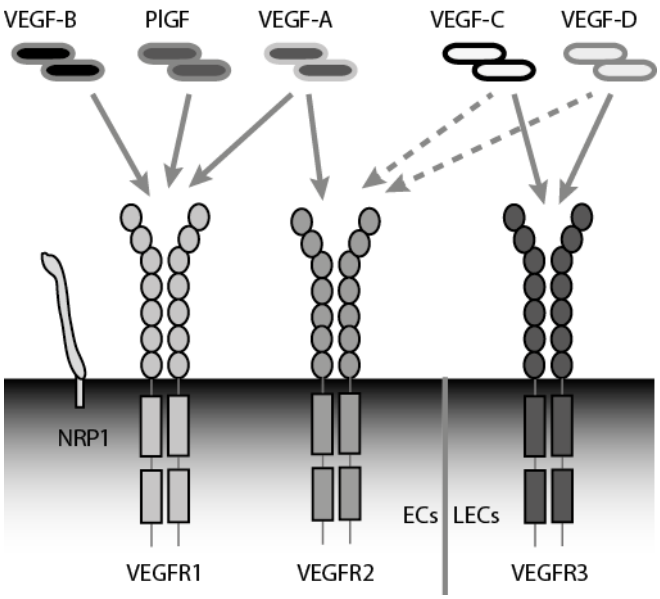



*Figure 2. VEGF ligands and receptors.* The five dimeric ligands bind to three receptors in a partially overlapping manner on endothelial cells (ECs) and lymphendothelial cells (LECs). Dashed line indicates that proteolytic cleavage is needed before receptor binding. Only ligand homodimers are shown. VEGF-B, PLGF and VEGF-A all bind NRP1 (binding pattern not shown).

## VEGF-A

VEGF-A (also called Vascular Permeability Factor, VPF) is the major angiogenic factor. It binds to VEGFR1, VEGFR2 and the NRPs, but exerts its main angiogenic functions via VEGFR2[15,23]. Its expression is strongly induced by hypoxia and Hypoxia Inducible Factor 1$\alpha$ (HIF1$\alpha$)[24]. *Vegfa* expression is also regulated in a HIF-independent manner in response to nutrient deprivation[25] (see Chapter 4). The various VEGF-A splice isoforms have different binding affinities to HSPs and thus different solubility[26]. This creates a VEGF-A gradient, which endothelial tip cells of the existing vasculature can sense and migrate towards[27]. VEGF-A is a potent permeability factor[28], and a survival factor for the ECs[29].

VEGF-A is required for the development of the vascular system, as mice lacking even a single copy of the gene die *in utero* from defects in EC-development and vascular formation[30,31]. The highest *Vegfa* expression in the adult animal is found in heart, skeletal muscle and lung[23]. The expression of VEGF-A is also upregulated in most solid tumours, and in some other pathological conditions such as atherosclerosis, diabetic retinopathy and arthritis[23].

Several anti-angiogenic therapies targeting VEGF-A/VEGFR signalling have been developed[12,13]. The monoclonal antibody Bevacizumab (*Avastin)* that targets VEGF-A, as well as the TK-inhibitors Sunitinib (*Sutent*) and Sorafenib (*Nexavar*), have been US-FDA approved and are commonly used in the clinic for the treatment of several cancers[13]. All three drugs have multiple mechanisms of action including normalisation of the tumour vasculature and direct effects on the tumour cells[13]. Anti-VEGF-A therapy using Bevacizumab is also used for the treatment of proliferative diabetic retinopathy[32,33].

## VEGF-B

VEGF-B was discovered in Ulf Eriksson's laboratory in 1996[21]. The two splice isoforms, VEGF-$B_{167}$ and VEGF-$B_{186}$, differ in their C-terminal amino acid sequences[34], and show different diffusion properties and receptor-binding affinities[34,35]. Both isoforms bind specifically to VEGFR1 and NRP1[35,36]. VEGF-$B_{167}$ is the predominant form and binds to cell surface HSPs, making it less soluble[37]. VEGF-$B_{186}$ is freely diffusible, but requires proteolytic cleavage before binding to NRP1[34]. Both isoforms are expressed in adult tissues, with the highest expression in the myocardium, brown adipose tissue (BAT), skeletal muscle and pancreas[34,37]. During the development, *Vegfb* is expressed in the mouse heart and BAT, and the cardiac expression is strong already at day E8.5[20,38].

The VEGF-B knock-out mice are healthy and fertile, and do not show vascular defects[7,8]. Moreover, the expression of VEGF-B is not induced by hypoxia, in contrast to all the other VEGF-ligands[9,39]. The angiogenic role of VEGF-B has been studied in the context of ischemic injury, pulmonary hypertension and retinopathy, but the results have been both disappointing and contradicting[40-44]. Cardiac overexpression of VEGF-B by adenoviral delivery induced heart-specific angiogenesis[45,46], whereas genetic overexpression in the hearts of transgenic mice did not lead to any vascular changes[47]. The consensus is that VEGF-B has a restricted role in angiogenesis[45-48].

Instead, the overexpression of VEGF-B seems to robustly induce myocardial hypertrophy[45,47,49,50]. The molecular mechanism behind this remains unclear. The hypertrophy requires cardiac expression of VEGFR1, and could therefore involve paracrine signalling between ECs and cardiomyocytes[45]. Alternatively, it could be due to excess delivery of nutrients to the tissue, as mice overexpressing VEGF-B have increased cardiac ceramide accumulation[23,47]. The hypertrophy has been explained by expression of *Vegfr1* on cardiomyocytes[49,50], which is in contrary to previous reports[19,51]. It is important that the correct expression pattern of VEGFR1 is resolved, in order to fully understand the function of VEGF-B. Finally, it was shown that VEGF-B has anti-apoptotic effects on neurons in the brain and retina, by downregulating pro-apoptotic proteins belonging to the BH3-only family[52,53]. In conclusion, the biological role of VEGF-B signalling to the endothelium has remained enigmatic due to contradictory results and the lack of identified endothelial downstream targets[54].

## Placental Growth Factor (PlGF)

PlGF is, similarly to VEGF-B, a VEGFR1-specific ligand, and can also bind to the NRPs[55-57]. Although PlGF is expressed at low levels in the healthy adult, its expression is strongly upregulated in different pathologies such as tumour growth and atherosclerosis[14,58]. PlGF stimulates vessel growth, either directly or by enhancing VEGF-A-driven angiogenesis (Figure 3), and it also recruits other pro-angiogenic cell types such as macrophages[14,59]. PlGF knock-out mice have no apparent morphological changes, but the mice show weaker tumour growth and delayed wound healing mechanisms, further supporting a pathological role for PlGF[60-62]. PlGF has therefore been considered as an ideal anti-angiogenic target for cancer treatment, and results from tumour-bearing mice receiving anti-PlGF antibodies initially looked very promising[63-65]. However, recently the efficiency of anti-PlGF treatment was questioned, and further research on the role of PlGF in vascular growth is needed[66].

## VEGFR1 and VEGFR2

VEGFR2 (KDR or Flk-1) is the main angiogenic and vasculogenic VEGF-receptor[15,23]. *Vegfr2* is expressed post-developmentally by proliferating ECs and tumour ECs and by neurons, but is downregulated in the quiescent endothelium[15,67]. VEGF-A binds directly to VEGFR2 and induces subsequent signalling through either PI3K or MAPK mediated pathways[23]. VEGF-C and VEGF-D can also bind to the VEGFR2 after proteolytic activation[23]. Interestingly, the affinity of VEGF-A is greater towards VEGFR1 then VEGFR2[15,17] (Figure 3).

The role of VEGFR1 (Flt-1) in angiogenesis is less clear. *Vegfr1* is expressed by ECs[51], monocytes and macrophages, and in adults it is mainly involved in vascular permeability and inflammatory cell recruitment[59,65]. VEGFR1 is also produced as a secreted, soluble form (sVEGFR1), which can act as a trap for all its ligands during vessel growth, and in the placenta[68]. During development VEGFR1 seems to attenuate VEGF-A/VEGFR2 signalling by trapping VEGF-A (Figure 3)[69]. *Vegfr1*$^{-/-}$ embryos die early during development due to overgrowth and derangement of vessels[69]. On the contrary, deletion of only the intracellular TK-domain of VEGFR1 (VEGFR1-TK$^{-/-}$ mice) results in healthy and fertile mice, which have reduced transendothelial migration of macrophages[70]. Difficulties in detecting receptor phosphorylation in response to VEGF-A has added to the confusion around VEGFR1[15,23]. This has lead to the "sink theory", discussed on the next page.

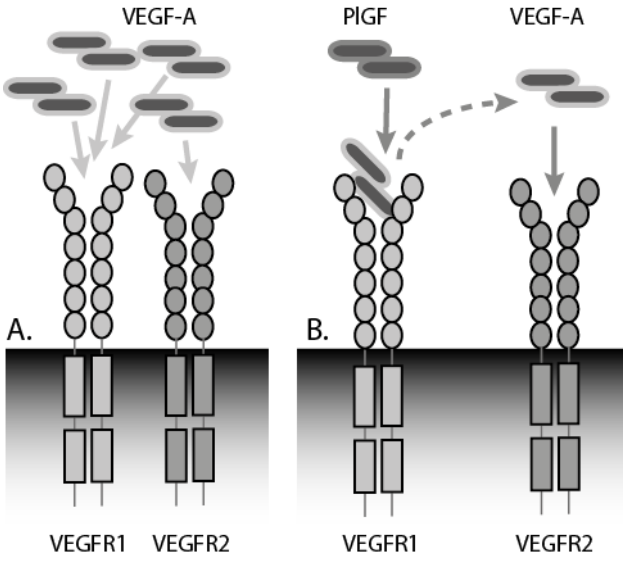



Figure 3. Schematic drawing of the sink theory. A) VEGF-A has higher affinity for VEGFR1 then VEGFR2, which leads to trapping of VEGF-A, and attenuation of the VEGFR2-mediated angiogenic signal. B) PlGF indirectly stimulates pathological angiogenesis by displacing VEGF-A from VEGFR1, thereby enabling VEGF-A/VEGFR2 driven angiogenesis.

## NRPs

The NRPs were originally discovered as receptors for semaphorins, mediating repulsive signals during axonal guidance[71]. It was long thought that NRPs do not possess signalling capacity themselves. Recently the C-terminal tail of NRP1 was shown to bind to and signal via PDZ-domain containing proteins[72]. This will open up for novel interesting research, as focus has previously been on the detection of VEGFR-phosphorylation levels in response to ligand binding.

The NRPs have a wide expression pattern, and are found in both the nervous and the vascular systems[15]. NRP1 is expressed by the arterial vasculature, and also by many tissue cells such as cardiomyocytes and myocytes, whereas NRP2 is more prominently expressed by the venous and lymphatic endothelium[15]. *Nrp1*$^{-/-}$ mice die prenatally due to defective neuronal patterning and vessel formation[73], whereas *Nrp2*$^{-/-}$ mice survive development but also have neuronal defects[15,74,75].

### 1.1.2 VEGFs: controversies and recent advances

Although the angiogenic field has made several significant advances over the last ten years, there still exist some interesting unanswered questions.

*Ligand redundancy and specificity*. How does a redundant ligand/receptor system manage to produce ligand specific signalling? The "holy grail" of the VEGF-field is still unresolved. PlGF and VEGF-A signalling have been shown to phosphorylate separate sets of tyrosine residues on VEGFR1, and thereby induce growth factor-specific downstream target genes[60]. It would be interesting to perform large-scale analysis comparing the transcriptional and post-transcriptional changes in response to all three VEGFR1 ligands, including VEGF-B. Specific co-receptors can be enough to induce ligand specific signalling. This has been shown for other redundant growth factor systems, namely the novel FGFs[76]. The discovery that NRP1 has signalling capacity might open for similar findings within the VEGF-family. NRP1 has been shown to, independently from VEGFR2, mediate VEGF-A-stimulated migration of ECs[77]. The identification of peptides or motifs important for binding NRP1 might lead to the identification of epitopes specific for each growth factor[18,78].

*The sink theory revisited*. According to the sink theory, the role of VEGFR1 is to attenuate VEGF-A/VEGFR2 signalling by trapping a portion of the secreted VEGF-A[48] (Figure 3A). The theory is based on the lack of angiogenic phenotype in the *Vegfb*$^{-/-}$ and VEGFR1-TK$^{-/-}$ mice, together with the strong binding affinity of VEGF-A to VEGFR1[7,8,60]. In addition, part of the pathological angiogenic effect of PlGF stems from displacing VEGF-A from VEGFR1, thereby increasing VEGF-A/VEGFR2 signalling[45] (Figure 3B). However, evidence is now emerging in support of VEGFR1-specific downstream signalling[45,52]. Presently it is thought that VEGFR2 mediates angiogenesis in response to acute stimuli, and is downregulated in quiescent vasculature[67]. In contrary, VEGFR1 and its ligand VEGF-B instead mediate the basal physiological functions of the vasculature, such as macrophage transmigration across ECs and nutrient uptake[47,59]. Therefore the *Vegfb*$^{-/-}$ and VEGFR1-TK$^{-/-}$ mouse phenotypes are subtle[48]. In addition, the biological roles of VEGF-B, PlGF and VEGFR1 might also be disguised by other redundant systems with overlapping functions.

## 1.2 FATTY ACID UPTAKE AND TRANSPORT

*"Defining the mechanism(s) of LCFA movement through membranes is vital to understanding whether or not entry of FA into cells can be controlled at the plasma membrane of a typical cell"*

Hamilton, Guo and Kamp[79]

Nutritional long-chain fatty acids (LCFAs) are delivered to peripheral tissues in two forms in the circulation, either as part of lipoprotein-associated triglycerides (TGs), or as albumin-bound non-esterified fatty acids (NEFAs). Lipoproteins are the major postprandial delivery route, whereas during fasting high circulating NEFA levels originate from lipolysis of the white adipose tissue (WAT).

In short, chylomicrons deliver TGs to peripheral tissues directly from the intestine, whereas Very Low Density Lipoproteins (VLDLs) are assembled in the liver from both chylomicron-remnants and from endogenous lipids[80,81]. TGs from both of these lipoproteins are then hydrolyzed at the site of peripheral tissues through the action of vascular bound lipoprotein lipase (LPL), yielding NEFAs[81]. Lipoproteins can also be endocytosed via binding to lipoprotein receptors, which are not discussed here in detail. The subsequent transport of NEFAs across the endothelium has been poorly characterised[3] as most research has been focused on sarcolemmal transport[82,83] (Figure 1).

Originally, it was suggested that hydrophobic NEFAs could freely diffuse through the plasma membrane (PM) by a process called flip-flopping[79]. Today, we have ample evidence supporting the idea that protein-mediated FA uptake is the major route of entry of NEFAs into cells[84]. The cellular transport of NEFAs is saturable and substrate selective. Treatment of cells with proteases, which hydrolyse all extracellular proteins, inhibits cellular FA uptake. Furthermore, the working tissue needs to ensure lipid delivery when circulating NEFA levels are low, and limit uptake when they are high, thus demanding an active transport system. Three groups of proteins have been implicated in FA transport over the PM[84]: CD36, Fatty Acid Binding Proteins (FABPs) and the Fatty Acid Transport Proteins (FATPs)(Figure 3). Most genetic knock-out studies of these proteins have yielded healthy, viable mice despite defective FA accumulation[84-86], supporting the existence of redundant pathways that rescue nutrient delivery.

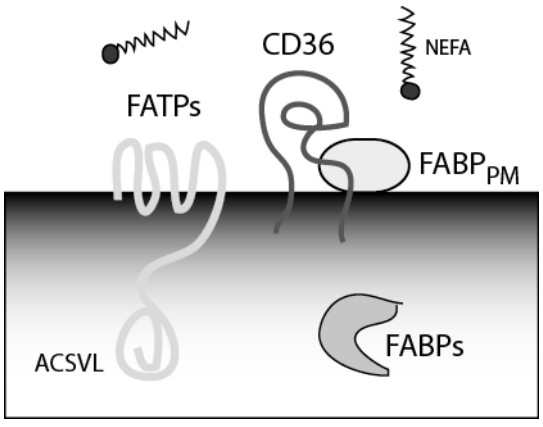



*Figure 4. FA handling proteins.* The FABPs are intracellular soluble proteins, whereas the FATPs are though to span the PM six times and CD36 has two transmembrane domains. FABP$_{PM}$ is associated to CD36 on the extracellular side.

### 1.2.1 Scavenging fatty acids: CD36

CD36 (also called Fatty Acid Translocase, FAT) is an 88 kDa, heavily glycosylated scavenger ("cleaning") receptor that belongs to an evolutionarily conserved protein family of scavengers[87]. CD36 recognises and binds to a large variety of lipids and lipid structures[88,89], and its multiple functions have led to four different lines of CD36 research. CD36 has a role in innate immunity as it recognizes bacterial lipid components, and participates in phagocytosis[87,88,90]. CD36 is also the endothelial receptor for mediating the anti-angiogenic signalling of thrombospondin-1[91,92]. CD36 is well studied as a macrophage scavenger receptor for oxidized low-density lipoproteins (LDL), and has been attributed a role in atherosclerotic lesion formation[93-95]. Additionally CD36 is studied as a facilitator of physiological LCFA-transport[96], which will be discussed here.

*Expression. Cd36* is expressed by platelets, monocytes, ECs and by parencymal cells in WAT, BAT, heart and skeletal muscle[87,97]. Its expression on ECs is still debated[98-100]. Importantly, *Cd36* is strongly upregulated by the nuclear transcription factor Peroxisome-proliferator activated receptor gamma (PPARγ)[93,101]. Endothelial-specific deletion of PPARγ in mice ablated endothelial *Cd36* expression, and in many aspects phenocopied the *Cd36*$^{-/-}$ mouse[102] (see below).

*Peripheral LCFA-uptake.* CD36 potently binds FAs and facilitates their uptake, although the detailed mechanism remains unknown and controversial[103-107]. CD36 localises to lipid rafts within the PM[108], and the mechanism might involve vesicle formation, or CD36 might work in tandem with other FA handling proteins (Figure 6). CD36 is activated both in response to glucose[109] and insulin[110], suggesting a postprandial role for CD36 in lipid uptake. It has also been show that CD36 can bind native lipoproteins[89] and participate in the clearance of chylomicron from the circulation[111]. In addition to mediating FA uptake in heart and muscle, CD36 is important for the intestinal absorption of nutritional fat[111]. For further discussion on this topic, see Chapter 4.

*Knockout studies. Cd36*$^{-/-}$ mice are viable and fertile, but show lower intra-muscular TG contents and lower blood glucose, and have hypertriglyceridemia due to reduced FA uptake[112,113]. Muscle-specific overexpression of CD36 results in muscular lipid accumulation and lower plasma VLDL levels[114]. It was concluded that inhibition of CD36 was highly beneficial. However, in a subsequent study when the mice were fed either a high sucrose or a high fat diet (HFD), the *Cd36*$^{-/-}$ mice responded *worse* then wt mice, and developed insulin resistance (IR)[115]. This was probably because global CD36-deficiency also affects the WAT, leading to ectopic lipid accumulation in the liver and other non-CD36-dependent organs[116] (see Chapter 3). Moreover, it was discovered that the Spontaneously Hypertensive Rat (SHR) acquires its phenotype due to a CD36 deficiency[117,118]. The SHR has been used as a model for a model for type 2 diabetes (T2D), hypertriglyceridemia and defective lipolysis. The finding of numerous CD36 deficient patients, with symptoms ranging from T2D to cadiomyopathy, further strengthens the importance of adequate CD36 expression[119,120].

*Role of CD36 in WAT*. *Cd36*$^{-/-}$ mice have defective FA uptake to the WAT and reduced fat mass[112,113]. The mice have constantly higher plasma TGs, whereas their NEFAs are only elevated during fasting, suggesting defective WAT-lipolysis and/or LPL-function instead of reduced NEFA uptake[113]. CD36 deficient SHR rats have *larger* adipocytes then control strains, suggesting that CD36 is not simply involved in adipocyte lipid accumulation[117]. Thus, CD36 has an important role in FA accumulation in WAT, although the mechanism remains unknown.

*PPARγ*. The thiazolidinediones are PPARγ agonists that have been used as anti-diabetic drugs[121]. They are thought to induce PPARγ-mediated shunting of TGs from peripheral tissues to the WAT, thereby increasing peripheral insulin sensitivity[122] (see Chapter 3). CD36 is upregulated by PPARγ[104]. As the thiazolidinediones therefore *induce Cd36* transcription, increased CD36 levels, especially in the WAT, should be beneficial. Exploring the consequences of CD36 overexpression in adipocytes or in the WAT vascular bed, and comparing the results to treatment with thiazolidinediones, would be extremely interesting. The association of both human and rodent CD36 deficiency with numerous pathologies[123] indicates that proper CD36 expression is important, especially in the WAT, and further ideas of therapeutic *inactivation* of the protein should be limited[124].

### 1.2.2 Intracellular FA handling: the FABPs

The FABPs consist of at least nine ~15 kDa mammalian members with different expression patterns and biological functions[125]. They are soluble cytoplasmic proteins that traffic their ligands between various intracellular compartments. The most well studied FABPs are the adipose FABP (A-FABP or aP2), and the epidermal FABP (E-FABP), which both also are expressed by ECs[126] (Chapter 4). Most of the FABPs have been genetically deleted[125]. The genetic studies underline the idea that altered intracellular lipid distribution has major impacts on systemic metabolism.

In contrast, the $FABP_{PM}$ (Got2 or mAspAt) does not belong to the same FABP family[84]. It is larger (40 kDa) and associated with both the PM and CD36 on the extracellular side (Figure 4), and is also found in mitochondria[84]. Its primary role thus seems to be to assist CD36 in FA-handling[84].

### 1.2.3 The enigmatic FATPs

The FATPs[127] (Fatty Acid Transport Proteins or Slc27a-family) are a family of six mammalian proteins (FATP1-6) which transverse the PM several times[128] (Figure 4). The 70-80 kDa large proteins are evolutionary conserved[129] and expressed in a tissue- and/or cell type-specific manner[86]. However, their specific detection is difficult due to a high sequence similarity in their C-terminus, towards which most antibodies classically are made (AF, unpublished observation). Their mechanism of FA transport is not known, although it has been proposed that FATP1-dimers can form a pore through the PM[130,131].

The FATPs have an intracellular (Very) Long chain Acyl-CoA Synthase (ACS(V)L) domain[132]. There has been much debate as to whether the FATPs are true LCFA-transporters, or if they simply drive cellular FA influx by acylation[133,134]. Yeast has been used as a model since it has one FA transporting FATP-orthologue, and one PM-associated ACSL. It has been suggested that during evolution, the two yeast-proteins have merged into one mammalian FATP protein[135]. By using yeast strains deficient of their endogenous FA transporter, and transfecting them with the mammalian FATPs, it was demonstrated that FATP1, FATP2 and FATP4 readily induce FA uptake, whereas the other members had weaker activity upon overexpression[136]. All FATPs except for FATP5 could also acylate the FAs. These types of studies could be complemented by using combinations of different FATPs, in order to detect enhanced activity through heterodimerisation.

Another controversy surrounding the FATPs has been the apparent intracellular localisation of FATPs to the Golgi network[137] or to the endoplasmic reticulum[138]. Despite of the localisation, the FATPs potently induced NEFA uptake. The use of green fluorescent protein fusion proteins and cross-reactive antibodies in these studies cannot explain the results completely. It has been proposed that the FATPs localize to transport vesicles, which are found intracellularly when the cells are not actively taking up LCFAs[139]. FATP1 and FATP4 have been shown to be transported to the PM of adipocytes in response to insulin stimulation[139,140]. Time-lapse studies of different cell types that are stimulated with various NEFAs could be very informative, in order to better understand the role and intracellular localisation of all the FATP members.

**FATP1** has a similar expression pattern as CD36 and is found on white and brown adipocytes, myocytes and cardiomyocytes[134] (Figure 6). We found FATP1 to be expressed by some but not all endothelial cell lines, but not in isolated primary cardiac ECs (Paper I). FATP1 requires homodimerisation for proper FA uptake, although this might not be unique to FATP1 as dimerisation has not been studied for the other FATPs[130]. One report showed that *Fatp1*$^{-/-}$ mice had normal weight gain and plasma triglycerides, in sharp contrast to *Cd36*$^{-/-}$ mice[141]. It further demonstrated that *Fatp1*$^{-/-}$ mice have less intramuscular FAs and greater insulin sensitivity, but only after a HFD[141]. In contrary, a second study reported decreased basal FA uptake in the *Fatp1*$^{-/-}$ mice, and protection against HFD-mediated weight gain[140]. *Fatp1*$^{-/-}$ animals also have defective thermogenesis in response to cold exposure[142]. FATP1 is not considered to be important in cardiac FA accumulation, but forced cardiac overexpression of FATP1 results in lipid-induced cardiomyopathy[143]. In conclusion, FATP1 is a potent FA transporter, but the organ-specific roles of FATP1 in FA uptake are still under investigation.

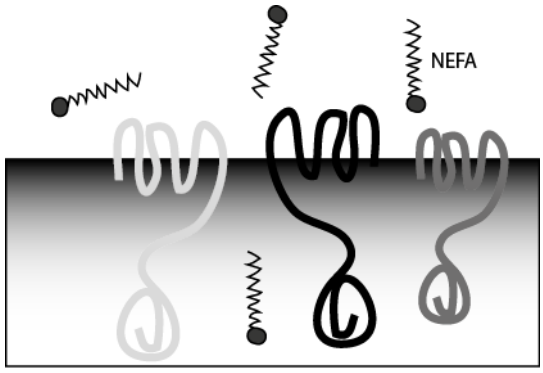
**FATP2** is expressed in the liver and the kidney[86]. The protein localises both to hepatic peroxisomes and to the PM, and FATP2 has a function within hepatic FA transport[144].

**FATP3** has not been thoroughly studied. Overexpression of FATP3 in yeast showed that FATP3 has ACSVL activity, but not FA transporting activity[145]. *Fatp3* is widely expressed, including by some endothelial cell lines[97].

**FATP4** has a similar expression pattern as FATP1, but is also expressed in the skin[134]. Three groups independently showed that *Fatp4*$^{-/-}$ mice die at different stages during development or at the latest post-natally due to severe dermal defects[146-148]. The mice have defective formation of the dermal barrier resulting from reduced ACSVL activity, and the phenotype is completely rescued by keratinocytic expression of FATP4[149]. Moreover, it has been shown that mutations in *Fatp4* can cause an inherited skin disease in humans[150]. The embryonic lethality of the *Fatp4*$^{-/-}$ mice has restricted *in vivo* research.

**FATP5** is the liver-specific FATP, and is expressed post-developmentally on the PM of hepatocytes and possibly also in hepatic ECs[151]. *Fatp5*$^{-/-}$ mice have decreased hepatic lipid uptake and accumulation, but instead shunt lipids to heart, muscle and WAT, and the resulting ectopic lipid accumulation led to hyperglycaemia[151]. Feeding the *Fatp5*$^{-/-}$ mice a HFD led to lower food intake and increased uncoupled thermogenesis, and subsequently less weight gain[152]. The underlying reason might have been defects in lipoprotein output from the liver to the WAT[151].

**FATP6** was described as a heart-specific FATP[153], but this has since been disputed[134]. FATP6 readily transports LCFAs, and immunohistochemical localization indicated both sarcolemmal and endothelial localization within the heart[153].




FATPs on the cell surface

## 1.3 CURRENT VIEW OF METABOLIC DISEASE AND T2D

*"One of the great ironies of the present-day industrialized world is serious disease and death brought about by too much rich food and too little physical exertion"*

*Rosen and Spiegelman*[101]

Over the past 10 years the view of the causes and mechanisms behind T2D and metabolic disease has radically changed[154]. It is only now being recognised that the major T2D-preceding event, insulin resistance (IR), is the underlying cause of the disease. From being called "a sugar disease" T2D is nowadays considered to be a lipid-associated disorder, where hyperglycaemia develops as a consequence of IR, and not as the cause of T2D[5,155,156]. Understanding why and how different lipid species influence insulin sensitivity is therefore of vital importance.

### 1.3.1 Pathophysiology of T2D

*Type 1 diabetes* (T1D, insulin-dependent or juvenile diabetes metillus) develops when the β-cells within the pancreatic islets are attacked and destroyed by the patient's own immune system. T1D is an autoimmune disease whose pathologic origin lies within the pancreas, and the disease usually becomes apparent when the patient is fairly young. Islet destruction severely decreases insulin production, and without insulin tissues cannot efficiently take up glucose. As a consequence, hyperglycaemia develops, and the patients need to substitute with exogenous insulin. Poorly managed T1D can lead to the development of micro- and macrovascular complications, as well as metabolic derangements such as ketoacidosis[157].

*Type 2 diabetes* (T2D, non-insulin dependent or adult-onset diabetes) is currently the most common form of diabetes and comprise 95% of diabetes patients[158]. T2D shares most complications with T1D, but the aetiology is very different. In T2D the β-cells of the pancreas are initially capable of synthesising and secreting insulin, but the peripheral tissues such as liver, muscle, heart and WAT cannot sense and respond to the hormone[159]. This *decreased insulin sensitivity* deteriorates and subsequently develops into total IR. Whereas T1D patients usually are otherwise healthy, T2D is strongly associated to obesity and other metabolic diseases (Figure 5).

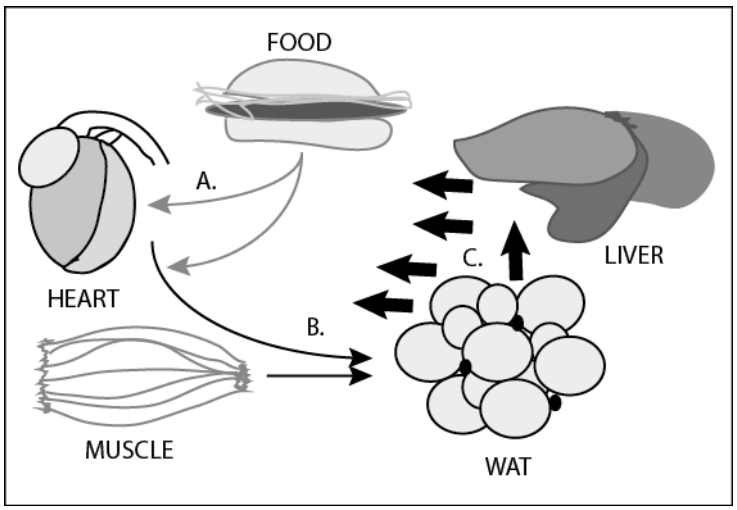



*Figure 5. The path to ectopic lipid accumulation.* Nutritional FAs are taken up in the intestine and distributed to the working tissues (A), and excess lipids are stored in the WAT (B). An imbalance between work and nutrition, together with a maximised storage capacity in WAT, leads to spill-over of FAs from WAT to peripheral organs (C).

As the peripheral IR increases, several fatal changes occur:

1 **Muscle:** Decreased insulin sensitivity leads to less glucose uptake. This is compensated by increased lipid uptake and oxidative metabolism[160].
2 **Pancreas**: To compensate for IR in peripheral tissues, the pancreas increases insulin production. This leads to pre-diabetes characterized by islet hyperplasia and circulating hyperinsulinemia[156]. The patients become diabetic when the peripheral need for insulin exceeds the capacity of the pancreas. The pancreatic β-cells cannot sustain high insulin production, and start to deteriorate, whereby circulating insulin levels drop. Severe islet deterioration leads to insulin-dependent T2D[156,161].
3 **Plasma:** Glucose start to accumulate in the bloodstream.
4 **Liver:** Insulin inhibits gluconeogenesis in the liver and increases glycogenesis, thereby limiting hepatic glucose output. The liver is usually one of the first organs to become IR. Hepatic IR results in continued glucose secretion, which further fuels the developing hyperglycaemia[162].
5 **WAT**: Lipolysis is normally also inhibited by insulin. As a consequence of IR, the WAT continues to secrete NEFAs. The NEFAs stay in circulation, and are also taken up by the liver. Severe hepatic lipid accumulation can lead to triglyceridemias and fatty liver disease. Additionally, insulin-induced TG-uptake to the WAT is decreased in IR.
6 **Dyslipidemia**. Increased lipolysis from the WAT and decreased adipocyte uptake of lipoproteins results in high circulating lipid levels, and contributes to the risk of atherosclerotic and other cardiovascular diseases.
7 **Related pathologies**: T2D patients have increased risk of cardiovascular disease, stroke, amputation, blindness and kidney failure. Most of these associated pathologies are common for both T1D and T2D, and their underlying molecular mechanisms remain controversial[154,163]. They will not be further discussed within this thesis.

### 1.3.2 Aetiology of T2D

IR in multiple organs and cell types lead to the development of T2D. But what causes cells to become insulin resistant? Strong evidence suggests that IR is caused by excessive lipid accumulation within non-adipose tissues[5,156,164] (Figure 5). For example, intramyocellular TG content in humans is a determinant of IR[165]. The infusing of fatty acids into lean, healthy subjects acutely impairs glucose uptake[5]. Various mechanisms for *how* lipids can cause IR have been proposed, and it is unlikely that only one mechanism is involved. The list below includes some of the most interesting past and present theories.

#### Contributing factors

*Thrifty genes*. Evolution has programmed us to efficiently accumulate all available nutrients during times of plenty, meaning we are in constant preparation for periods of fasting. The thrifty gene theory, laid out by James Neel almost fifty years ago, proposes that some of our more "greedy genes" have today become detrimental, causing the metabolic syndrome[166,167]. Examples are the pro-lipogenic SPREB1c[168] and the upregulation of FA transporters in response to a HFD[160,169]. Given today's sedimentary lifestyle, we have not evolved to eat at McDonalds every day.

*WAT-spill-over effect*. Although obesity is strongly associated with T2D, evidence is emerging that it is the accumulation of fat *outside* the WAT that is most damaging[156,164]. This has elegantly been shown in genetic rodent models where the storage capacity of the WAT was expanded or restricted[164,170]. Overexpression of adiponectin in ob/ob mice increased the number of adipocytes, leading to WAT hyperplasia[170]. The transgenic mice became overtly obese, while maintaining normoglycaemia. Restricting the storage capacity of each adipocyte in another study resulted in lean, severely diabetic mice[164]. Therefore, a healthy hyperplastic WAT is the safest storage site for lipids, protecting peripheral tissues from ectopic lipid accumulation. Overriding the storage-capacity for individual adipocytes (hypertrophy) increases lipolysis and spill-over of the TGs (Figure 5).

*WAT inflammation*. Obesity has been shown to cause inflammation in the WAT[171]. This has a negative impact on peripheral tissues through increased secretion of pro-inflammatory cytokines like Tumour Necrosis Factor $\alpha$, and decreased secretion of beneficial adipokines such as adiponectin[171]. Whether WAT inflammation is a cause or consequence of IR within the tissue is still debated[172].

*Decreased insulin receptor signalling*. Several different lipid species have been shown to have negative effects on insulin mediated cellular glucose-uptake[5]. Through the use of genetic models, the laboratory of G.I. Shulman has demonstrated the adverse effects of diacylglycerols (DAG) on insulin receptor signalling. DAGs are ligands for the novel Protein Kinase C (PKC) $\theta$ and $\varepsilon$[173]. Activation of PKC $\theta$ alters the phosphorylation pattern of the Insulin Receptor Substate 1 (IRS-1), leading to decreased insulin receptor signalling. Ceramides, synthesised from saturated FAs, are another type of neutral lipid that have been implicated in cellular IR[174]. The exact pathological mechanism of the ceramides is however less well characterized[174].

*Defective Glut4 vesicle transport*. Excess accumulation of lipids in lipid droplets has been shown to counteract Glut4-mediated glucose uptake in response to insulin signalling[175,176]. The accumulation of lipid droplets within myocytes traps vesicle fusion proteins belonging to the SNARE-family in the cytoplasm[177]. This directly prevents SNARE-mediated fusion of Glut4 vesicles to the PM in response to insulin-stimulation. Interestingly, the same proteins have been implicated in the fusion of insulin-filled secretion vesicles to the pancreatic PM[178]. This mechanism could thereby explain the pancreatic insulin secretion defect that occurs in response to IR.

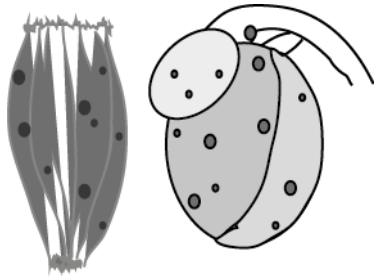
*Mitochondrial deficiency*. The expression of mitochondrial genes is decreased in response to a HFD and in diabetic patients[179-181]. Recently, it was shown that saturated FAs can cause epigenetic changes on DNA, which reduce the expression of the transcriptional co-regulator PPAR$\gamma$ co-activator 1 $\alpha$ (PGC1$\alpha$). PGC1$\alpha$ is a master regulator of mitochondrial biogenesis, and decreased PGC1$\alpha$ expression leads to lower mitochondrial content and thereby less mitochondrial oxidation of lipids[182]. Lower lipid oxidation could further fuel lipid accumulation, leading to a vicious cycle. However, it is debated whether mitochondrial dysfunction is a cause or a consequence of metabolic disease[183].

### Possible ways to resolve IR

*Increased lipid metabolism.* It has been hypothesised that enhancing lipid oxidation in IR-tissues could induce insulin sensitivity. However, an increase in FA-oxidation would also drive the formation of reactive oxygen species (ROS)[5]. Increased ROS can damage cell structures and lead to oxidative stress. Moreover, according to the first law of thermodynamics, fat cannot be lost without exercise or work[184]. The only way to oxidise lipids "for free" is by mitochondrial uncoupling, whereby heat is generated instead of ATP. Brown adipocytes are specialized at heat generation through uncoupling in response to cold, thereby oxidising large quantities of lipids[185]. Therefore, it is hypothesized that a brownification of the WAT, and/or increased lipid uptake and oxidation in the BAT, could present a future therapeutic opportunity[186].

*Decreased muscular FA uptake.* Almost all knock-out models of lipid handling proteins expressed in the skeletal muscle have increased glucose tolerance[116,141,187] (Chapter 2). This supports the notion that decreased lipid uptake and accumulation in skeletal and cardiac muscles is highly advantagous. The drawback of decreased muscular FA uptake is shunting of the lipids to other organs like liver, or increased hypertriglyceridemia like the in the *Cd36*$^{-/-}$ mice (Chapter 2). Ideally, the FA uptake to WAT should be increased simultaneously with decreased muscular FA usage.

In conclusion, novel research demonstrates that ectopic lipid accumulation leads to various metabolic derangements, although it is not yet agreed upon which cellular mechanisms or specific type of lipids cause IR[188]. Therapeutically the aim is to be able to control peripheral lipid accumulation, while shunting excess lipids to BAT for oxidation, or to WAT for safe storage. How such a lipid shunting is achieved is currently not known. However, if the endothelium has a regulating role in organ-specific lipid-uptake, this could open up novel opportunities of metabolic control.



Ectopic lipid accumulation

## 1.4 VASCULAR METABOLOMICS: THE FUSION OF VASCULAR BIOLOGY AND METABOLISM

*"During obesity induced by HF feeding, inflammation and insulin resistance develop in the vasculature well before these responses are detected in muscle, liver, or adipose tissue."*
Francis Kim et al 2008[172]

As described earlier, the vasculature has been widely studied in the context of tissue oxygenation[2], but not as much for its role in metabolic regulation. Conversely, metabolic researchers have often not considered the vasculature as a physiological barrier[82,83] (Figure 1). Times have changed and a range of novel publications has recently addressed the role of the vasculature in different aspects of metabolic physiology and pathology.

### 1.4.1 Endothelial insulin response and IR

Previously it was believed that insulin induces endothelial permeability, and that the resulting increase in local vascular leakage allowed for insulin and nutrients to pass through the vascular wall[189]. Similarly, it has been proposed that FAs, released from lipoproteins by LPL, induce local vascular leakage in order to access the PM of the parenchymal cells where FA transport proteins are expressed[81,190]. Nevertheless, evidence has emerged that insulin is actively transported through the endothelium, and that this transport is the rate-limiting step in muscular glucose uptake[191,192].

Insulin-stimulation of ECs induces two major changes to the microvasculature. Firstly, insulin increases nitric oxide (NO) production[192]. NO is a vessel relaxant, which dilates the vessels and increases the blood flow through the capillaries. This increase in capillary surface area is important for endothelial glucose uptake. Knock-out mice of the endothelial NO synthase (eNOS) have reduced glucose uptake and insulin sensitivity due to their inability to produce NO in response to insulin[193]. Secondly, insulin also regulates the transport of both itself and nutrients through the ECs[191]. Knock-out mice lacking IRS-2 specifically in ECs have reduced delivery of insulin to muscle cells, and significantly decreased glucose uptake. The authors also showed that endothelial NO production is regulated through IRS-2-mediated insulin signalling[194]. Based on these results, the endothelium should be added to the list of insulin sensitive organs.

Insulin sensitivity is decreased upon excessive lipid exposure (Chapter 3). Recently, it was shown that endothelial IR *precedes* the IR of myocytes. After feeding mice a HFD for one week, endothelial Akt phosphorylation and NO production in response to insulin were attenuated[172]. Myocytes and hepatocytes became measurably IR only three weeks later, whereas the WAT developed IR even later[172]. Thus, endothelial IR is emerging as a novel component of the metabolic syndrome, joining the ranks of the other components listed in section 1.3.1[195].

### 1.4.2 Metabolic coupling of the tissue and vasculature

If metabolite uptake to muscle is regulated at the level of the endothelium and not the sarcolemma, how can parenchymal cells assure sufficient nutrient supply to support their metabolic needs? Control of nutrient uptake is especially important in light of the deleterious effects of excess intra-muscular lipid accumulation (Chapter 3). Thus, there must be a communication between tissue cells and the vasculature, coordinating energy needs to nutrient uptake. This communication can be called *metabolic coupling* of the vasculature and the surrounding tissue.

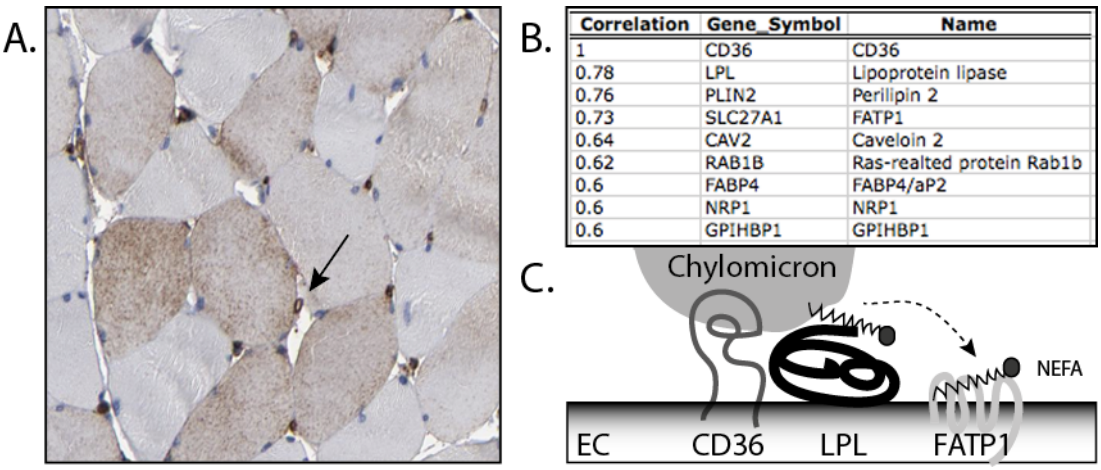
LPL is an interesting example of metabolic coupling (Chapter 2). LPL resides on the luminal side of the endothelium, where it hydrolyses lipoprotein-associated TGs. However, it is not synthesised by ECs, but by parenchymal cells[81]. LPL is then transported through the endothelium to the vascular wall by an EC-specific transport protein, glycosylphosphatidylinositol-anchored HDL-binding protein 1 (GPIHBP1)[81,196]. Knock-out of GPIHBPI leads to severe triglyceridemia due to low endothelial-bound LPL levels. The trans-endothelial transport of LPL allows for tissue cells to maintain transcriptional control of *Lpl* expression and lipoprotein-hydrolysis, whereas the vasculature controls LPL-transport to the vascular wall.

Metabolic coupling of the vasculature to the tissue also helps the working muscle to adapt to a specific nutritional need or increased workload. Fasting leads to a rapid induction of a complete pro-angiogenic transcription program, including increased *Vegfa*-expression[25]. This is mediated by PGC1$\alpha$ and the transcription factor Estrogen Related Receptor $\alpha$ (ERR$\alpha$). Similarly, $\beta$-adrenergic signalling in response to exercise increases PGC1$\alpha$-levels in skeletal muscle, and leads to VEGF-A synthesis and exercise-induced angiogenesis[197]. Both exercise- and fasting-induced angiogenesis are HIF1$\alpha$-independent, and the authors speculate that physiological angiogenesis could be differentially regulated compared to pathological angiogenesis[25,197]. Cold exposure has also been shown to mediate a VEGF-A driven angiogenic program in WAT and BAT, which is HIF1$\alpha$-independent but requires VEGFR2[198]. It would be very interesting to compare the transcriptional and post-transcriptional changes in ECs undergoing physiological and pathological angiogenesis.

### 1.4.3 Permeability barrier for nutrient-uptake

The role and mechanism for vascular LCFA uptake has been poorly studied. Several FA handling proteins have previously been indicated to be expressed by the endothelium. These include CD36[199], FATP1[3], FATP5[151], FATP6[153], FABP3[200], FABP4[126] and FABP5[201]. Many reports are regarded as circumstantial by the research field, and detailed uptake mechanisms for endothelial FA uptake, and FA transport through the endothelium, remains enigmatic[3,202]. It seems that the expression of *Cd36, Fatp1* and potentially also *Fabp4* is co-regulated (Figure 6B), and these FA handling proteins could be cooperating both on the endothelium and on the parenchymal cells (Figure 6a).

*Potential mechanism of CD36-mediated FA-uptake.* Based on the CD36 literature review presented in Chapter 2, a novel hypothesis on the role of CD36 in endothelial FA uptake will be described below. Hypothetically, CD36 could be involved in the binding and localisation of TG-rich lipoproteins to the vascular wall, so that LPL efficiently can hydrolyse them (Figure 6C). CD36 is transported to the PM in response to insulin, suggesting a postprandial function[110]. Lipoproteins have been shown to bind to regions rich in lipid rafts[81], and CD36 is similarly associated with these regions[108]. Data from publicly available databanks support the endothelial localisation of CD36 in muscle[203], and shows a strong correlation[204] of *Cd36*-expression with both *Lpl*- and *Gpihbpi*-expression (Figure 6). A role of CD36 in tethering lipoproteins would explain why the *Cd36*$^{-/-}$ mice had elevated TGs instead of elevated NEFAs[113], and go well with the reported function of CD36 in the clearance of chylomicron[89,111] (see Chapter 2). Additionally, a recent paper showed CD36 activity to be vital for the uptake of TG-rich lipoproteins to BAT in response to cold exposure[103]. This FA handling complex is probably found both on the ECs and on the sarcolemmal PM. Hopefully, future research will help to confirm or deny this theory, taking us one step closer to a full understanding of endothelial lipid uptake.




| Correlation | Gene_Symbol | Name |
|---|---|---|
| 1 | CD36 | CD36 |
| 0.78 | LPL | Lipoprotein lipase |
| 0.76 | PLIN2 | Perilipin 2 |
| 0.73 | SLC27A1 | FATP1 |
| 0.64 | CAV2 | Caveloin 2 |
| 0.62 | RAB1B | Ras-realted protein Rab1b |
| 0.6 | FABP4 | FABP4/aP2 |
| 0.6 | NRP1 | NRP1 |
| 0.6 | GPIHBP1 | GPIHBP1 |

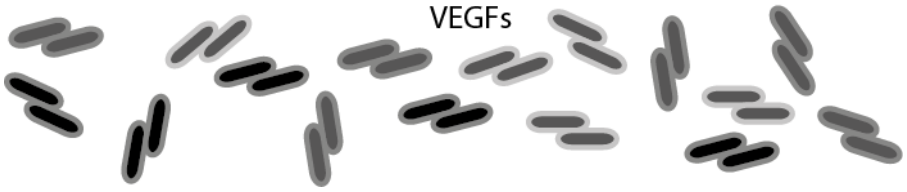
Figure 6. Hypothetical novel role for CD36. A) Immunohistochemical staining for CD36 in human muscle taken from the Human Protein Atlas. Arrow indicates vascular localization. B) Shortened list of genes correlating with CD36 in the Sym Atlas database. C) Schematical illustration of the hypothesis. CD36 binds to chylomicrons in the circulation, and theters them to the vascular wall so that LPL can hydroslyse associated TGs. FATP1 could also belong to this functional unit.

*VEGF-A and glucose uptake.* The endothelium expresses at least two facilitating glucose transporters, *Glut1* and *Glut3*, although *Glut1* is far better characterized then Glut3[205]. Interestingly, some reports suggest that VEGF-A can upregulate Glut1 expression in ECs, at least in the blood-brain barrier[206,207]. Circulating VEGF-A levels are also correlated with lower blood glucose levels in humans[208]. Positron Emission Tomography (PET) measuring $^{18}$F-deoxyglucose ($^{18}$FDG) uptake has been used in the clinic to detect glycolytically active tumours and metastasis in cancer patients[209]. When the $^{18}$FDG uptake in specific tumours was compared to the expression of *Vegfa* and *Vegfb* within the tumours, the results showed that glucose uptake correlates positively with *Vegfa* expression and negatively with *Vegfb* expression[209]. These results can be

explained both in the context of hypoxia-dependent and hypoxia-independent regulation of *Vegfa* expression. Hypoxia is associated with a non-oxidative, glycolytic metabolism[210]. However, during oxidative metabolism glucose is also needed to replenish the tricarboxylic (TCA) cycle.

VEGF-A has also been shown to upregulate the expression of *Fabp4* in endothelial cells *in vitro*[126]. This regulation was VEGFR2-dependent, and inhibition of FABP4 decreased the proliferation of the ECs, suggesting that FABP4 is needed for efficient cell division. Similarly, VEGFR1 has been shown to be activated by LDLs, leading to endothelial internalization of the lipoprotein and increased cholesterol supply to the endothelium[211]. Both these events have been connected to the need for structural lipids and cholesterol in an actively dividing vasculature.

In conclusion, the vasculature is of vital importance not only for deliveries of nutrients and oxygen, but also for helping the tissues to control their energy metabolism and adapt to physiological changes in their environment. VEGF-A is a potent mediator of fasting- and exercise-induced angiogenesis, and VEGF-A signalling to the endothelium may upregulate various nutrient transporters. Additionally, PlGF has been implicated in adipogenesis, although its roll within the WAT is unclear[212-215]. Thus, there is an emerging role within vascular metabolomics for members of the VEGF-family of growth factors.



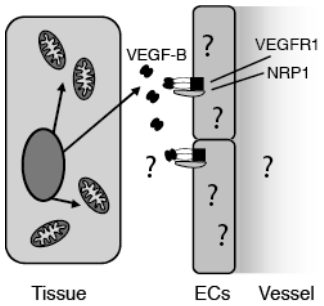
# 2 AIMS OF THE THESIS WORK

The biological role of VEGF-B-signalling has remained enigmatic. The starting point of this thesis work was bioinformatics showing a strong correlation between the expression of nuclear-encoded mitochondrial genes and *Vegfb*.

The aim of this thesis work was to characterise the biological function of VEGF-B in physiology and pathology, with special attention to metabolism.

The specific aims were:

- To characterize the role of VEGF-B in FA-metabolism, and how VEGF-B function is connected to vessel physiology (Paper I).
- To identify endothelial downstream target genes for VEGF-B, and to examine the metabolic phenotype of the Vegfb$^{-/-}$ mice (Paper I)
- To study the effects of genetic deletion of VEGF-B in a mouse model of T2D (Paper II)
- To investigate if treatment with neutralising antibodies against VEGF-B could alleviate IR and T2D (Paper II)



# 3 RESULTS AND DISCUSSION

### 3.1.1 Paper I: VEGF-B controls fatty acid uptake.

The biological role of VEGF-B has remained enigmatic, as previous studies of *Vegfb*$^{-/-}$ knock-out mice did not reveal any vascular defects or abnormalities (Chapter 1). We therefore used bioinformatics to identify links between VEGF-B and novel signalling pathways, metabolic networks or cellular functions **(Figure 1a in Paper 1)**. Genes with similar expression patterns were clustered together using a large set of publically available microarray data. Surprisingly, *Vegfb* readily clustered (r = 0.90) with a group consisting of only nuclear-encoded mitochondrial genes. Most of these were so-called OXPHOS genes[179], coding for proteins with functions within the oxidative phosphorylation chain, and in particular within Complex I.

We found more evidence supporting the co-regulation of *Vegfb* and OXPHOS-genes **(Figure 1b-d)**. *Vegfb* is strongly expressed in tissues with a high mitochondrial content, such as heart, oxidative skeletal muscle and BAT[20]. The expression pattern of *Vegfb* is similar to the expression of two markers for the OXPHOS genes, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5 (*Ndufa5)* and Cytochrome C somatic (*Cycs)*. The expression of *Vegfb*, *Ndufa5* and *Cycs* was equally decreased in response to overnight fasting, and increased in response to a HFD. We further confirmed the bioinformatics by using the mouse SymAtlas database[204] to search for genes co-regulated with *Vegfb*. By using a cut-off of 0.80, we found that only 31 genes were co-expressed with *Vegfb*, whereas many more genes were co-expressed with *Vegfa* and *Plgf*. The low number of genes suggested that the expression of *Vegfb* is tightly regulated. We further discovered that the co-regulation of *Vegfb* and OXPHOS genes had previously been indicated in a larger study characterising mitochondrial proteins[216]. This provided an independent validation of the results.

The tight co-regulation between the OXPHOS genes and *Vegfb* stems from transcriptional control by ERR$\alpha$ and PGC1$\alpha$ (X.W. and U.E., manuscript in preparation). Interestingly, the same transcriptional machinery also regulates the expression of *Vegfa*[25]. The correlation between *Vegfa* and mitochondrial genes is much weaker (r = 0.30) as compared to *Vegfb* **(Figures 1c and S1)**. Moreover, *Vegfa* expression is induced by fasting[25], whereas fasting seems to reduce *Vegfb* expression *in vivo*. These differences underline a unique role of *Vegfb*, but also point out that the metabolic regulation of the VEGFs still demands further investigation.

The bioinformatics studies indicated that VEGF-B has a role in energy metabolism. Considering that VEGFR1 is only expressed by ECs within tissues[19,51,69] (Chapter 1 and **Figure S10**), it suggested that VEGF-B could be helping the vasculature to sense the energy status of adjacent parenchymal cells. Hypothetically, higher PGC1$\alpha$/ERR$\alpha$ transcriptional activity would lead to coordination of mitochondrial biogenesis and VEGF-B secretion, which would be sensed by ECs through the signalling of VEGFR1. The model became complete when we found that the downstream endothelial effectors were members of the FATP family (Chapter 2 and Figure 7).

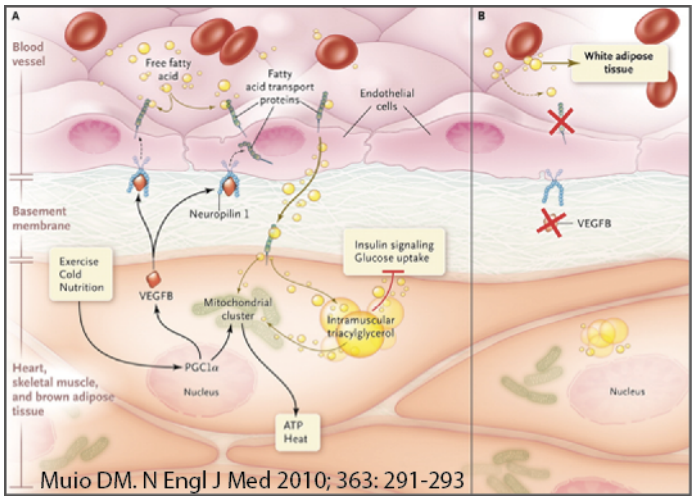



*Figure 7. Schematic summery of Paper I featured in NEJM 2010.* In Panel A, VEGF-B mediates FA-uptake through transcriptional regulation of FATPs. In panel B, FAs are instead shunted to WAT when VEGF-B is missing. Reproduced with permission of the publisher.

Many aspects of FATP biology were unknown (see Chapter 2). Which of the FATPs, if any, were expressed by the endothelium *in vivo*? Could all FATPs facilitate LCFA transport? Were the vascular FATPs required for endothelial LCFA uptake and transport?

We found that VEGF-B signalling through VEGFR1 and NRP1 on ECs could control the transcription of specific FATPs both *in vitro* and *in vivo* **(Figures 2-3)**. The change in relative mRNA expression of each FATP was modest, around 2.5-3 fold, which was reflected by a similar change in *in vitro* LCFA uptake upon VEGF-B stimulation. However, the siRNA experiments confirmed that the FATPs were necessary and sufficient for LCFA uptake *in vitro*. In terms of which of the FATPs was the most important for FA transport regulated by VEGF-B, the results pointed towards FATP3. The expression of *Fatp3* was dependent of VEGF-B signalling in all tested cell lines, tissues and animal models except for in the lung **(Figure 2, S4, S5 and S9)**. Although *Fatp3* is expressed at low levels, its expression seems to be specific to the vasculature. We showed for the first time that FATP3 required co-expression with FATP4 in order to be fully active **(Figure 3d)**. Homodimerisation has been reported for FATP1 (Chapter 2), making the necessity for FATP3/FATP4 heterodimers an interesting hypothesis. This could be one of the reasons why FATP3 has not previously been considered to facilitate FA uptake[145].

The tissue and cell specificity of VEGF-B/FATP-regulated LCFA uptake is very interesting **(Figure 3, S6, S11 and S16)**. The lack of effect of VEGF-B signalling in isolated primary cardiomyocytes and brown adipocytes, as well as in cultured HL-1 cardiomyocytes is most probably due to that the cells lack VEGFR1[45]. However, treatment with VEGF-B did not induce transcription of *Fatp4* in VEGFR1-expressing NIH3T3 fibroblasts. Moreover, we found no transcriptional changes of *Fatp3* in isolated primary lung ECs or in the small intestine from *Vegfb*$^{-/-}$ mice. Similarly, another research group failed to find changes in the transcription of FATPs in the pancreas of transgenic mice overexpressing VEGF-B specifically in the pancreatic β-cells[217]. Possibly we have yet to identify some critical component for VEGF-B/FATP function downstream of VEGFR1, which has a restricted expression pattern that varies between different vascular beds.

We currently believe that FATP transcription cannot be the only pathway for vascular LCFA uptake controlled by VEGF-B (A.F. and U.E., unpublished data), and ongoing research aims to identify additional transcriptional or post-transcriptional components. The FA uptake phenotype of *Vegfb*$^{-/-}$ mice seems more robust than the modest changes in FATP transcription, supporting additional mechanisms. A re-examination of the metabolic needs and preferences of different tissues, as well as further research of how such a preference is controlled, would be highly interesting.

We confirmed the novel role of VEGF-B *in vivo* by examining the metabolic phenotype of *Vegfb*$^{-/-}$ mice **(Figure 4)**. To measure organ-specific LCFA uptake we fed the mice $^{14}$C-labeled oleic acid by oral gavage. However, there are some drawbacks with this method. It does not allow for examination of the intracellular destination within an organ. It is also difficult to show whether VEGF-B-mediated uptake is more important for chylomicron-, VLDL- or albumin-bound lipids. Cellular β-oxidation will not damage the radioactive labelling, but entry into the TCA cycle will rapidly transfer $^{14}$C to $CO_2$, making detection of FA uptake impossible after exhalation. One would therefore like to develop new methods using more sensitive and specific labels, as was recently done in one study[103].

The oral gavage experiments showed that the labelled LCFAs in *Vegfb*$^{-/-}$ mice accumulated in various WAT depots after 24 hours. The *Vegfb*$^{-/-}$ mice gained more weight with age, and their increased adiposity was confirmed by magnetic resonance imaging. The shunting of FA uptake to WAT should be beneficial for whole body metabolism, but normally lead to undesired hypertrophy (Cahpter 3). Surprisingly, the adipocytes of the *Vegfb*$^{-/-}$ mice were instead hyperplastic (A.M. and U.E., manuscript in preparation). The molecular mechanisms behind increased lipid accumulation and hyperplasia in *Vegfb*$^{-/-}$ WAT are still unknown. No major differences in WAT *Fatp3* and *Fatp4* expression were detected, but *Fatp1* expression was increased in *Vegfb*$^{-/-}$ WAT (A.M. and U.E., unpublished data). Adiponectin was not changed[170]. Ongoing research includes using metabolic cages to examine eating behaviour, activity and the respiratory quotient of the *Vegfb*$^{-/-}$ mice, in order to build a complete picture of the factors influencing weight gain.

To confirm that the decreased muscular LCFA uptake in Vegfb$^{-/-}$ mice had an impact on lipid accumulation, we used Oil Red-O (ORO) staining of frozen tissue sections **(Figure 4e-g)**. ORO is a classical method that stains neutral lipids within tissues, but does not detect biological membranes[218]. However, this method is not without disadvantages. It is tedious and requires analysis of multiple microscopic frames. The ORO staining can vary considerably between different regions of an organ. Additionally, it unspecifically stains all neutral lipids, not distinguishing between different lipid species. FATPs have been shown to have the highest affinity for different types of LCFAs. In our *in vitro* assays we used 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-S-indacene-3-dodecanoic acid (BODIPY-FA), a saturated 16-carbon LCFA mimic, and oleic acid, an 18:1-unsaturated FA. Alitalo and co-workers reported accumulation of ceramides, synthesised from saturated FAs, in the hearts of transgenic mice with cardiac-specific VEGF-B overexpression[47]. It would therefore be interesting to discover if the VEGF-B/FATP system has a preference for saturated LCFAs, and if this could explain the beneficial effects of targeting VEGF-B (Paper II). Given the

recent advances in technology, large-scale metabolomics using HPLC/MS or magnetic resonance spectroscopy to investigate intra-muscular lipid accumulation would be the most appropriate method to assay for lipid accumulation within *Vegfb*$^{-/-}$ tissues. We hope that such technology in the future will help us to identify what kind of lipids the VEGF-B/FATP system transports.

Lastly, the results should be discussed in the context of VEGF biology **(Figure 2).** The bioinformatics and *in vitro* cell culture experiments suggested that the role of VEGF-B in oxidative metabolism is unique and different from the other VEGFR ligands. Importantly, experiments done by us and others[45] using neutralising antibodies against VEGF receptors showed that inhibiting VEGFR2 has no effect on VEGF-B signalling. This is in contrast to part of the angiogenic affect by PlGF with is mediated by displacing VEGFR1-bound VEGF-A (Figure 3). The FATPs provide a novel target gene downstream of VEGFR1 signalling since VEGFR1-TK$^{-/-}$ mice had reduced cardiac FATP expression. Identification of the FATPs will permit assays where PlGF- and VEGF-B-dependent activity can be monitored and separated. We addressed the ligand redundancy by adding a 10x excess of PlGF together with VEGF-B$_{186}$, but there was no change in VEGF-B signalling. This occurred despite the protein's ability to bind to the same domain of the receptor[15,18]. It suggests that the two VEGFR1-specific ligands VEGF-B and PlGF recognise individual epitopes on the receptors, or the involvement of an additional co-receptor. Additionally our results show that proper signalling by the VEGFR1 intracellular domain is required for adult physiological FA uptake.

In conclusion we report an unexpected and novel role for VEGF-B in controlling vascular LCFA transport. This role is specific for VEGF-B within the VEGF family of growth factors, and requires proper function and endothelial expression of VEGFR1 and NRP1. VEGF-B signalling to ECs induces transcriptional upregulation of members of the FATPs, most notably *Fatp3*. This leads to transport of LCFAs across the vascular barrier in a manner proportional to FATP expression. As a consequence, *Vegfb*$^{-/-}$ mice and NRP1-EC$^{-/-}$ mice have lower cardiac accumulation of neutral lipids. We could further show that *Vegfb*$^{-/-}$ mice instead have higher mobilisation of glucose uptake into their hearts, suggesting a compensatory switch in metabolism. The co-regulation of a vascular growth factor with OXPHOS genes suggests a novel regulatory mechanism that exists to keep working muscle supplied with adequate amounts of nutrients (Figure 8). Thus, VEGF-B has a role in metabolic coupling of the tissue cells and the endothelium.

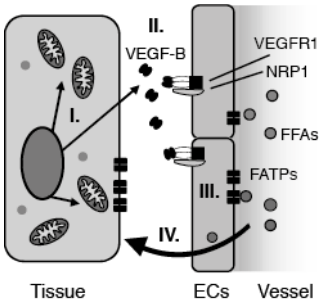



*Figure 8. Schematic illustration on the role of VEGF-B*: I) VEGF-B is co-expressed with mitochondrial proteins in order to coordinate lipid uptake and β-oxidation. II) VEGF-B signals in a paracrine fashion to endothelial VEGFR1 and NRP-1. III) Stimulation of ECs with VEGF-B upregulates vascular FATPs, and (IV) subsequent transport of LCFAs across the EC layer.

### 3.1.2 Paper II: Targeting VEGF-B in IR and T2D

IR, T2D and its complications are becoming an increasing health problem worldwide[158]. Lifestyle changes are still by far the most effective treatment for T2D[219]. It is now recognised that IR and intra-myocellular lipids are the major underlying causes for the development of T2D-associated pathologies (Chapter 3). Although the precise cellular mechanism and the type of lipids are not agreed upon by the field, it is clear that intra-myocellular lipids should be targeted in both pre-diabetic and diabetic subjects[5].

With this background, we asked whether targeting the VEGF-B/FATP axis could decrease intra-myocellular lipid accumulation in a pathological context as well. We aimed to study this by genetically deleting *Vegfb* and by targeting VEGF-B therapeutically using a neutralising antibody treatment. As an animal model, we chose to use the *db*/*db* mouse, which is on of the most well-characterised and severe animal model of T2D presently available[220].

The lack of co-regulation between *Vegfb* and mitochondrial OXPHOS genes in *db*/*db* mice raised many interesting questions (**Figure 1**). Mitochondrial content is reduced in response to obesity, IR and T2D[180] (Chapter 3). In line with this, we found reduced cardiac expression of *Ndufa5* and *Cycs* in the diabetic *db*/*db* mice as compared to healthy *db*/+ controls. To our surprise, the expression of *Vegfb* as well as *Fatp3* was not reduced in the diabetic mice. Similarly it has been proposed that obese men have higher circulating VEGF-B levels as compared to lean men[221]. However, questions remain as to whether this dysregulation between *Vegfb* and OXPHOS expression is specific for *db*/*db* mice, wheter it can be found in other diabetic models, and most importantly in diabetic patients. It does correlate well with the induction of *Cd36* and *Fatp1* expression in response to T2D[98,222]. The regulatory mechanism remains unknown, although epigenetic changes could be involved (Chapter 3). Thus, future research will have to clarify how *Vegfb* expression is regulated in response to a HFD, IR and T2D.

To study how genetic deletion of *Vegfb* affected the development of T2D in *db*/*db* mice, we crossed them with *Vegfb*$^{-/-}$ mice (**Figure 2-3**). We found that in contrast to *db*/*db* controls, *db*/*db*//*Vegfb*$^{-/-}$ and *db*/*db*//*Vegfb*$^{+/-}$ mice did not develop hyperglycaemia, and they maintained weight gain throughout the study. We confirmed that the heterozygous *db*/*db*//*Vegfb*$^{+/-}$ mice maintained the expected level of *Vegfb* expression in the heart. It will be interesting to complete these results with similar analyses of other organs, most notably skeletal muscle. The reason for the heterozygous *db*/*db*//*Vegfb*$^{+/-}$ mice having such a strong phenotype thus remains unknown. Heterozygous *Vegfa*$^{+/-}$ and *Vegfc*$^{+/-}$ mice also have phenotypes[15] (Chapter 1). It might therefore be common for all VEGFs that tight expressional regulation is essential.

We next analysed the overall cardiac metabolism of the *db/db//Vegfb*$^{-/-}$ mice (**Figure 2**). *Db/db//Vegfb*$^{+/-}$ and *db/db//Vegfb*$^{-/-}$ mice have reduced *Fatp3* levels in the heart, and ORO staining showed reduced cardiac lipid accumulation upon *Vegfb* deletion. PET analyses showed that *db/db//Vegfb*$^{-/-}$ mice have increased basal cardiac glucose uptake. We confirmed these results by measuring total $^{18}$FDG radioactivity in isolated skeletal muscles and hearts using a dose calibrator. An identical increase in $^{18}$FDG radioactivity in both hearts and muscles from *db/db//Vegfb*$^{-/-}$ mice suggested that the phenotype of skeletal muscle is similar to that of heart, in accordance to what we found in Paper I. Although we have focused most experiments on the heart, future research will include further analyses of skeletal muscle, liver and pancreatic functions.

Total deletion of VEGF-B in the *db/db* mice was also associated with previously unidentified defects. The *db/db//Vegfb*$^{-/-}$ mice are very nervous and stressed, especially upon fasting. Handling of fasted animals, for example giving a sham PBS injection to a fasted *db/db//Vegfb*$^{-/-}$ mouse, acutely raised its blood glucose levels, whereas it did nothing to *db/db* controls or lean wt mice. The heterozygous *db/db//Vegfb*$^{+/-}$ mice have a more subtle stress response, and we have not observed any stress symptoms in animals treated with the anti-VEGF-B antibody 2H10. Presently, we believe that this is connected to some previously unknown developmental defects due to *Vegfb* deletion. It could potentially be associated with the anti-apoptotic role of VEGF-B on neuronal cells[52,53]. However, the glucose-liberating phenotype suggests increased cortisol secretion and/or sensitivity.

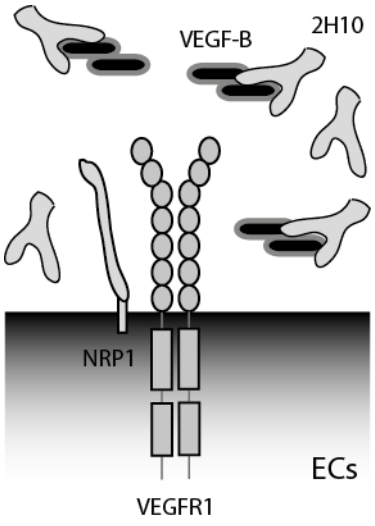
The glucose tolerance tests (GTT) and insulin tolerance tests on the *db/db//Vegfb*$^{-/-}$ mice showed that the mice have increased glucose tolerance and insulin sensitivity as compared to *db/db* mice (**Figure 3**). Enhanced glucose clearance during the GTT further suggested that mice lacking *Vegfb* maintain better pancreatic islet function, although we found no difference in basal insulin levels. Measurement of insulin secretion during the GTT would be highly informative. During the GTT, the blood glucose of the *db/db//Vegfb*$^{-/-}$ mice initially rose abnormally high, and several reasons for this could be plausible. IR might persist in the liver or in the WAT, where *Vegfb* is not highly expressed. The abnormal stress response could also be an influence. Future plans include analysing IRS-1 phosphorylation levels in various organs in order to characterise organ-specific IR.

To study the potential of VEGF-B as a therapeutic target, we took advantage of the previously described monoclonal antibody 2H10 (**Figure 4-5**). 2H10 was shown to have high affinity specifically for VEGF-B[223], and in Paper I we demonstrated that 2H10 prevented VEGF-B-mediated upregulation of FATPs *in vitro*. We have now shown that treatment of *db/db* animals with 2H10 for 10 weeks reduces cardiac *Fatp3* mRNA expression and lipid accumulation as visualised by ORO staining. Furthermore, 2H10-treatment of either pre-diabetic or diabetic db/db mice has clear anti-diabetic effects, suggesting that the antibody is able to traverse the vascular wall and neutralise VEGF-B found on the interstitial side. This is also in line with the results from Paper I showing acutely increased intra-cardiac lipid levels after adenoviral VEGF-B administration. Moreover, the finding that the heterozygous *db/db//Vegfb*$^{+/-}$ mice have a strong phenotype might explain the observed rigid response to antibody treatment.

The pre-diabetic mice treated with 2H10 did not develop hyperglycaemia, and had a normal GTT response at the end of the treatment period (**Figure 4**). The already diabetic mice receiving 2H10 showed a significant reduction in blood glucose levels as compared to controls and had enhanced glucose tolerance (**Figure 5**). Interestingly, neither of the 2H10 treatments resulted in a significant gain of body weight. We have not yet measured energy expenditure, activity and food intake in *db*/*db* mice treated with 2H10. Food intake is a critical factor when using a mouse model that has a targeted disruption of the leptin pathway.

Finally we analysed metabolic plasma parameters in the transgenic and antibody-treated *db*/*db* mice (**Figure 3-5**). The *db*/*db*//*Vegfb*$^{-/-}$ mice as well as the pre-diabetic 2H10-treated animals had reduced circulating levels of plasma NEFAs, TGs, ketones and low-density lipoprotein-associated cholesterol. The change in metabolic plasma parameters was as expected milder in the diabetic *db*/*db* mice receiving 2H10 as compared to the pre-diabetic mice, but still showed the same tendency. This suggests that reducing peripheral IR has positive effects on dyslipidemia and ketogenesis. The reduced levels of NEFAs might be due to the increased anti-lipolytic effect of insulin on WAT. This would also reduce the hepatic lipid accumulation, possibly leading to decreased synthesis of VLDL and cholesterol. Insulin signalling also inhibits ketogenesis, suggesting increased hepatic insulin sensitivity in response to 2H10 treatment. Taken together, the above assumptions might explain the significant changes in plasma metabolites. However, peripheral insulin sensitivity in specific organs has to be validated by analysis of organ-specific phosphorylation patterns and metabolism.

In conclusion we show that VEGF-B has the potential to be a novel target in the treatment of peripheral IR and T2D. We show that acute and genetic inhibition of VEGF-B signalling has significant anti-diabetic effects. Furthermore, 2H10 treatment also has profound effects on circulating metabolites, and leads to reduced triglyceridemia and ketosis. We therefore propose that inhibition of VEGF-B signalling is a novel exciting treatment option against IR and T2D.



# 4 FUTURE PERSPECTIVES

The studies included in this thesis will hopefully be the exciting starting points of much research to come. Some key questions are listed below.

Paper I contributed to the VEGF field by describing a novel metabolic role for a VEGF. Paper I also further connects FA uptake to tissue oxygenation by showing that growth factors of the same family can facilitate these two key events. Many interesting questions remain. It is still not known how the ligand-specific effects are mediated within the VEGF family. The discovery of a way to assay for VEGF-B signalling will facilitate further research on this topic. There exists many unknown intracellular steps downstream of VEGFR1 and NRP1 signalling. Furthermore, one should explore additional connections between the metabolic functions of VEGF-A and VEGF-B. Both VEGFs are regulated by the same metabolic transcription program including PGC1$\alpha$ and ERR$\alpha$ (Chapter 4). However, VEGF-A is also regulated by hypoxia, and can possibly induce glucose uptake. Clearly there are some interesting vascular metabolomics questions yet to be answered.

Secondly, Paper I and II both highlight the role of the vasculature as a potent barrier for nutrient uptake, and hopefully this will lead to more research exploring this topic (Chapter 4). What is the source of the lipids that are transported through the VEGF-B/FATP system, i.e. are they derived from lipoproteins and/or lipolysis or both? What is the role of the FATPs within ECs, and what is the transport route through the endothelium? Do perivascular cells such as pericytes or smooth vascular cells also contribute to nutrient uptake? Is glucose uptake regulated at the level of the vasculature in muscular tissues? Some recent results suggest this (Chapter 4). Additionally a re-evaluation of lipid transport over the blood-brain-barrier would be very interesting. Looking at these seemingly metabolic questions from a vascular perspective will hopefully produce much interesting research in the future.

Finally, it is clear that the results presented in Paper II are exiting and should be further expanded. The study still lacks some supporting evidence, such as measurement of the insulin sensitivity in individual organs and detailed characterisation of the role of VEGF-B in pancreatic function. In a long-term perspective, these results will be repeated in other animal models, and the toxicology of 2H10 should be investigated, hopefully in preparation for moving into pre-clinical trials. We are currently also working on a more complete phenotypic description of the *Vegfb*$^{-/-}$ mice, which will help to decipher developmental defects of VEGF-B-inhibition from acute effects. Thus research into the role of VEGF-B in fatty acid uptake and metabolic disease has only started, and hopefully we have managed to contribute to the emerging field of vascular metabolomics!

Lastly I would like to thank all of you who have actually read through this thesis, for your admirable interest and sincere dedication.

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